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13. ABSTRACT (Maximum 200) The erbB family of receptors and their cognate ligands, the neuregulins, have been implicated in a number of human malignancies. We have generated a mouse model of breast cancer by targeting the expression of one of these molecules, neuregulin $\beta 2c$, to the mammary gland. The results suggest that neuregulin can induce a variety of developmental and differentiative effects in the breast. These effects include the inhibition of terminal differentiation of the ductal tree, hyperplasias, and eventual tumor formation. When mated with another line of transgenic mouse overexpressing the c-myc gene, tumors of the breast are formed more rapidly, presumably through the inhibition of Myc-induced apoptosis. We have also used homologous recombination to generate mice lacking a functional c-neu allele. These mice are embryonic lethal, with aberrant neuronal development. We believe that cardiac development is unaffected in these animals, though it is a lack of cardiac trabecular development that has been suggested by others to be the cause of death.				
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FOREWORD

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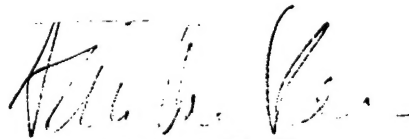
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Ian M. Krane, Ph.D.

PI - Signature

Date

Table of Contents

	Page
Introduction	2
Body	6
Conclusions	12
References	15
Figure Legends	20
Table	24

Introduction

Breast cancer is the most prevalent common form of cancer in women. To understand better the underlying cellular and molecular biological factors which lead to breast cancer, our laboratory has developed mouse models with which to study this disease(1-4). These models involve targeted expression of different oncogenes to the breast eventually resulting in tumorigenesis.

In recent years, a family of stimulatory ligands for the EGF/*erbB* family of receptor tyrosine kinases has been described. This family includes Neu differentiation factor (NDF), heregulin (HRG), acetylcholine receptor inducing activity (ARIA), and the glial growth factors (GGFs)(5-8). This family of ligands, known collectively as the neuregulins (NRGs), is comprised of alternatively spliced isoforms of a common gene. Interaction of the products of this gene with heterodimers and/or homodimers of the *erbB* receptors leads to their auto and trans-phosphorylation and subsequent interaction with SH-2 proteins including phospholipase C- γ (PLC- γ), the p85 subunit of phosphatidylinositol 3'-kinase (PI-3 K), and the GTPase activating protein of *ras* (*ras*-GAP)(9).

The EGF receptor (EGFR) has been associated with cancers of the breast, bladder, lung and stomach. Another member of the EGFR family, HER2/*erbB2*/*c-neu*, has been implicated in a large proportion of human malignancies, including breast and colon carcinomas, and its amplification and/or overexpression has been associated with a poor prognosis(10). Our laboratory has demonstrated that the overexpression of an activated form of this receptor, when targeted to the murine mammary gland using the mouse mammary tumor virus promoter (MMTV), is transforming(3). Overexpression of the cellular form of this receptor in the mammary gland is also transforming, though in a more stochastic fashion(11).

Binding of NRG to the cell surface through one or more of the *erbB* receptors is thought to stabilize receptor dimers, similarly to the effect produced by the point mutation found in the transmembrane domain of the activated *neu* oncogene, a mutation not found in human tumors(12). Although the role of NRG in breast cancer is still unclear, its biological effects on mammary epithelial cells *in vitro* are quite profound. Some mammary epithelial cell lines can be stimulated to proliferate more rapidly, while others enter G₀ arrest in response to NRG, and begin to exhibit markers of mammary cell differentiation such as casein and lipid production(5, 13). At

the molecular level, NRG can stimulate tyrosine phosphorylation of p185^{erbB2/neu}, though this phosphorylation is thought to occur via a trans-phosphorylation event in a ligand-induced heterodimeric complex (14). NRG has no affinity for p185^{erbB2/neu}, but it has been shown to lead to p185^{erbB2/neu} tyrosine phosphorylation through interaction with the receptors p180^{erbB3} and p180^{erbB4} (15-17) even though NRG does interact with homodimers of p180^{erbB3} (18) and p180^{erbB4} (17). Although p180^{erbB3} has little intrinsic tyrosine kinase activity in an insect cell expression system (19), it is constitutively phosphorylated when overexpressed in mouse fibroblasts (20). Explanations for the differential phosphorylation of the different *erbB* receptors focus on heterodimerization events and receptor crosstalk. p185^{erbB2/neu} and p180^{erbB3} cooperate to increase phosphorylation of p180^{erbB3} in mouse fibroblasts (21). It is also quite likely that the structural complexities of the products of NRG gene play an important role in the tissue specificity of ligand-receptor function (22). In fact, in mouse keratinocytes that express *erbB2/neu* and *erbB3* (but not *erbB4*), p180^{erbB3} responds mitogenically to NRG beta isoforms, but is unresponsive to alpha isoforms (23). Elevated expression of NRG in a number of human mammary tumor cell lines has been shown to correlate with the expression of vimentin, a marker of metastatic disease; and it has also been demonstrated that the human breast epithelial cell line, MCF-7, which is unable to form tumors in nude mice, will form tumors when stably transfected with NRG, the growth of which can be inhibited by anti-NRG antibodies (R.Lupu, personal communication).

Among our goals as put forth in the initial Statements-of-Work, we wished to generate enough recombinant NRG to attempt to dissect the biological mechanism(s) by which NRG can be both differentiative and proliferative to breast cancer cells. We had hoped to use a panel cell lines established in our laboratory. Unfortunately, it proved to be quite difficult in our hands to generate sufficient quantities of soluble NRG so we halted that part of the project to pursue others. We created a transgenic mouse model to test if the overexpression of NRG in the mouse mammary gland can lead to the transformation of the mammary epithelium, or lead to other, differentiative, effects on mammary gland development. Much of that work has been published(24).

The third part of this proposal addresses the role of the *neu* gene in development by targeted disruption of the gene. During development, the *neu* gene has been demonstrated to be widely expressed in epithelial organs. In rat and human fetal tissue, immunohistochemical staining had localized Neu protein to cells of the gastro-intestinal and female reproductive tracts, kidney, and lung, but not to the heart, musculoskeleton, or in endocrine or hematopoietic organs(25-27). More recently, however, erbB2 staining has been described in the heart(28). Again, weak or no staining had been seen in the fetal central nervous system, though targeted disruption of the gene does lead to lack of development of the trigeminal ganglion(28). Although we believed the targeted disruption of this gene might ultimately shed light on the development of breast cancer, it would ultimately give us insight into the role of this gene in the normal development of the embryo. Using whole-mount *in situ* hybridization, we have demonstrated a clear Neu expression in the developing embryonic mammary epithelium. In *Drosophila* and other organisms, *c-neu* homologs have been demonstrated to have a variety of roles in development(29-31). Currently at least four *neu*-related genes have been described in higher eukaryotes (*erbB-erbB4*)(32-35), and the targeted disruption of two of these genes and their cognate ligand, has led us to now believe there may be little functional redundancy among these molecules, as their disruption appears to lead to similar, but distinct, phenotypes(28, 36, 37).

In the last year and a half of our proposal, we have been examining the potential cooperative effects of the neuregulin transgene with a *c-myc* transgene. It has been suggested that another *erbB* ligand, transforming growth factor alpha (TGF- α) can cooperate *in vivo* to accelerate tumor formation(38). This cooperativity is thought to work through an inhibition of *myc*-induced apoptosis in these tumors. To date, NRG has been shown to inhibit and stimulate apoptosis, with an apparent isoform-specific efficacy(39). To determine if NRG can work with *myc* to accelerate tumor formation, possibly through inhibition of apoptosis, we have generated a number of *myc*/NRG bitransgenic animals.

Lastly, using a commercially available anti-heregulin antibody, we made the observation that there are two immunoreactive proteins found in a breast cancer cell line which appear to be differentially compartmentalized within the cell. These proteins, based on the antibody recognition site, would represent cytoplasmic isoforms of neuregulin. Interestingly, the cytoplasmic

domain of this family of molecules is remarkably well conserved across species, yet this region of the molecule has yet to be attributed a function. As a final part of our proposal, we sought to identify and clone these molecules.

Body

Recombinant neuregulin expression

To evaluate the effects of NRG on the differentiation/proliferation of various mammary adenocarcinoma cell lines, we hoped to incubate purified protein corresponding to the bioactive EGF-like domain of NRG (NRGED) with target cell lines of human and murine origin in a range of concentrations and time periods(5, 13). Effects were to be monitored by examining changes in cell proliferation and the appearance of mRNAs for differentiated mammary cell markers (lipids, caseins) as well as gross morphological changes. Immunoprecipitation and Western blotting of cell lysates after treatment with NRG can also be performed using anti-phosphotyrosine antibodies to monitor phosphorylation of the activated Neu receptor and of its substrates in response to NRG(9, 13). The cell types' origin represented by many of the human cell lines available to our laboratory are unknown, but we do have a number of murine cell lines created in our laboratory whose genotype is known. These cell lines are derived from mammary gland tumors from transgenic mice overexpressing *myc*, *ras*, and the activated *neu* oncogene in the mammary gland using the MMTV promoter/enhancer. The individual phenotype of each parental tumor is quite distinct from the others in that, in multiparous females, the *neu* tumors involve the entire mammary epithelium, and form quite rapidly with kinetics suggesting *neu* expression alone is sufficient for malignant transformation of the breast(3). *ras* and *myc* tumors, however, arise stochastically and are not as widespread within the gland(1, 2, 4). Histologically, these tumors are also quite distinguishable(40) therefore we may expect to see a differential response to NRG treatment among the various cell lines. The effect of NRG on these cells may give us an indication of how to inhibit the growth of tumors expressing one or another oncogene, perhaps using NRG or an NRG-blocking antibody.

To isolate protein for these studies, a variety of *E. Coli* and eukaryotic expression vectors are available for the production of foreign proteins. A number of vectors may have to be tried due to the extreme variation in levels of expression, solubility, bioactivity and ease of purification of the protein desired. For this purpose, we amplified a 300 bp DNA fragment containing the bioactive EGF-like domain of NRG by the polymerase chain reaction

(PCR)(41). The PCR template was a plasmid containing the full length NRG coding region originally amplified from a *ras*-derived murine mammary gland tumor. We then subcloned the NRGED cDNA into a pFLAG *E. coli* expression vector. In this system, the foreign protein is fused to an eight amino acid FLAG epitope which is recognized by commercially available antibodies to aid in protein identification and purification(42). Similar plasmid constructs are being made using the pGEX *E. Coli* expression system in which the protein of interest is fused to the glutathione S-transferase (GST) coding sequence. Foreign proteins soluble in aqueous solutions can be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized glutathione(43). The pGEX family of vectors has been engineered such that purified protein may be cleaved from the GST carrier by digestion with site-specific proteases such as thrombin or blood coagulation factor X_a.

We also cloned NRGED into a pcDNA1-based eukaryotic expression vector containing an interleukin-4 signal sequence and a FLAG epitope fused in frame to the NRGED. COS-7 monkey kidney cells, transiently transfected with this construct and metabolically labeled with ³⁵S-methionine, demonstrate production of a proper-sized NRGED protein secreted into the medium which can be efficiently immunoprecipitated with an anti-FLAG antibody. This purified product is currently being analyzed for its ability to stimulate specific autophosphorylation of the Neu receptor. Unfortunately, after making each of the above constructs, it became clear that in our hands we are unable to generate adequate amounts of neuregulin for our studies. The product expressed in the eukaryotic vector did show low levels of bioactivity, but we chose to put off this part of the project in favor of others.

NRGβ2 transgene construct and tissue-specific expression

The second part of this proposal is to ask if overexpression of NRG directed to the mammary gland can lead to abnormal mammary gland development or malignancy. I plan to use transgenic mouse technology to overexpress *neu* differentiation factor in the mouse mammary gland in hopes of demonstrating a phenotype similar to the overexpression of the normal Neu receptor. If NRG can function as part of an autocrine or paracrine loop in the mammary gland to stimulate growth or differentiation, one would predict that aberrant expression of this protein will lead to mammary gland dysplasia or neoplasia. A recombinant plasmid containing the MMTV LTR fused to the

murine $\beta 2c$ NRG isoform cDNA was microinjected into the male pronucleus of a one-cell mouse embryo. Ligated to the MMTV promoter/enhancer is a 1.2 kb cDNA containing the entire coding sequence of murine $\beta 2c$ NRG isolated from a v-Ha-*ras* -induced murine mammary gland tumor using the polymerase chain reaction (PCR). This *ras*-induced tumor(2) was chosen as a tissue source of NRG as the original NRG clone described was isolated from a *ras*-transformed RAT-1 fibroblast cell line(44). To ensure proper expression of this cDNA, simian virus 40 (SV40) splicing and polyadenylation signals were added to the 3' end of the transgene construct (Fig. 1).

Three transgenic founder animals carrying the fusion gene were generated (TG.IJ, TG.IK, and TG.IM), and each animal passed the transgene to its progeny in a Mendelian fashion. The best characterized line, TG.IJ, will be described here.

The tissue-specific expression of the transgene was determined by Northern blot analysis of total RNA isolated from a variety of organs. The probe used in this analysis was an SV40-fragment specific to the transgene construct. The SV40 fragment, when used as a probe, gives similar results to the NRG probe in Southern (Fig. 1) and Northern analyses, though it is preferred to the NRG probe because it does not recognize the endogenous gene or the multiple endogenous NRG mRNA transcripts (data not shown). Interestingly, highest levels of transgene expression are seen in the salivary gland of virgin animals, a gland rarely transformed by overexpression of other oncogenes using the MMTV LTR (Fig. 2).

Harderian gland hyperplasia in NRG transgenic mice

Approximately 50% of all TG.IJ animals, male and female, exhibit a unilateral or bilateral exophthalmous resulting from a progressive enlargement of the Harderian gland, a tubuloalveolar gland located within the orbit of many terrestrial species, though absent in primates. This phenotype is observed as early as the time of weaning (3 weeks of age), and is coincident with transgene expression, as animals exhibiting unilateral exophthalmous show no detectable transgene expression in the unaffected contralateral gland, but demonstrate considerable levels of transgene messenger RNA in the affected gland, higher even than those seen in the mammary gland (Fig.3). After histologic evaluation, these enlarged Harderian glands are characterized as hyperplastic adenomas. While growing *in situ*, they do not invade the bone

of the surrounding orbit, and they fail to grow when transplanted into a syngeneic host indicating they are not transformed.

Terminal end bud structures persist in the mammary gland

We studied possible effects of transgene expression in the developing mammary gland by examining ductal morphogenesis in these animals. Although pregnancy and lactation stimulate the highest levels of transgene expression from the MMTV LTR in the mammary gland, we have demonstrated that the MMTV-NRG transgene is clearly expressed in virgin glands. To determine if there are any developmental abnormalities associated with transgene expression in these virgin glands, we prepared mammary gland whole mounts stained with carmine red alum to examine the growth of the mammary gland ductal tree (Fig. 4)(45). In the normal developing mouse mammary gland, as the animal passes through sexual maturity, ductal epithelial structures gradually fill the mammary fat pad in response to mesenchymal signals(46). The terminal end bud in the developing gland functions as a growth point, driving ductal morphogenesis by providing differentiated ductal and myoepithelial cells for the formation and elongation of secondary ducts. In a wildtype virgin female, these multi-layered terminal end bud structures have virtually disappeared by ten weeks of age, as the multiple layers of end bud cells undergo an apoptotic regression(47). We sacrificed a number of virgin transgenic female mice at various developmental stages and found that, although the mammary glands of younger animals appeared normal, glands from older animals (>10 weeks) had increased numbers of TEBs as compared with age-matched control animals.

Breast tumors in NRG transgenic mice appear stochastically

To examine the potential ability of the MMTV-NRG transgene product to elicit a tumorigenic phenotype in the mice, female TG.IJ transgenic mice were set up to breed continuously, upon reaching sexual maturity, in order to maximize expression of the transgene from the MMTV LTR. After the first round of pregnancy, animals were monitored weekly for the appearance of tumors. By 14 months of age, each animal in the study had developed at least one mammary gland tumor, with the median age of tumor onset being 357 days after birth (Fig. 5). Examined histologically, all tumors were similar in nature and distinct from other MMTV-oncogene-derived mammary gland tumors described previously in our laboratory(40). Each tumor was

characterized as an adenocarcinoma with a squamous cell component, highlighted by abundant keratinaceous debris. Northern blot analysis of total RNA from these tumors confirms high levels of transgene expression, though the mRNA source is naturally more homogeneous than the "unaffected" contralateral mammary glands typically used for comparison (Fig. 6).

These tumors continue to grow well when transplanted into the mammary fat pad of syngeneic hosts and can be adapted to cell culture conditions. One such cell line, IJ9921, was examined for *erbB* receptor expression and tyrosine phosphorylation status by immunoprecipitation and western blotting. Although each of the four known *erbB* receptors is expressed in this cell line, apparently only *erbB3* is phosphorylated (Fig. 7). *erbB3* is thought to have little intrinsic kinase activity in its human form, though it is active in rodent form due to amino acid changes in the tyrosine kinase domain. It remains to be determined if the other receptors may be involved in its transphosphorylation in these tumors.

The c-neu proto-oncogene is vital to murine development

In addition to the study of the biological effects of NRG in the mammary gland through stimulation of the *erbB* receptor complex, we were interested in the role of one of these receptors, *erbB2*, in development. For targeted disruption of the *c-neu* gene, a 129 murine genomic lambda phage library was screened with a full-length *neu*-specific probe radiolabeled with [α - 32 P]-dCTP by random priming. Two overlapping clones were identified. Each clone was approximately 15-16 kb and contained the same 5' end. One clone, λ 13, is approximately 500 bp shorter than the other, λ 21, and therefore the shorter clone was used to make the targeting construct in order to employ the flanking 500 bp as a probe for analyzing potential targeted ES cell clones. In short, a 9 kb NotI-XhoI fragment of clone λ 13 was inserted 5' of the neomycin resistance cassette of the pPNT vector(48), and on the 3'- side of the neo^r cassette was placed an 800 bp BamHI-EcoRI fragment. This construct removes from the *neu* gene a 2-3 kb genomic fragment containing the exon encoding the essential transmembrane domain of the *neu* receptor protein as well as flanking sequence encoding parts of the extracellular and cytoplasmic domains (Fig. 8A). This construct should produce a non-functional gene encoding a soluble extracellular domain only. After electroporation of embryonic stem cells with this construct linearized with NotI, double

selection in media containing G418 and FIAU will be performed and clones will be picked for analysis by Southern blot hybridization with the aforementioned 3'-flanking probe (Fig. 8A). Because of the loss of a HindIII site contained in that region of the gene deleted in the targeting construct, we would expect a shift in the size of the genomic fragment detected with the flanking probe upon restriction with HindIII. Indeed we do see a shift from a wildtype 9 kilobase fragment to a 13 kilobase targeted allele (Fig. 8B). Alternatively, PCR analysis with oligonucleotides contained in the 3' region of the *neo^r* cassette (the *neo^r* cassette is in the same transcriptional orientation as the *neu* sequences) and oligonucleotides in the 3' *neu* flanking sequence found in clone λ 21 but not in clone λ 13 will be performed. PCR analysis in this case is feasible because of the relatively short 3' *neu* flanking sequence in the targeting construct (800 bp) which should yield a PCR product of approximately 1.4 kb. An embryonic stem cell clone carrying the disrupted gene was injected into blastocysts and several chimeric animals were isolated. These animals carried the disrupted allele and were able to pass it through the germ line. Homozygous null mutants fail to develop past day 9.5-10.0p.c. and apparently express no full length Neu protein, while heterozygous embryos express half wild-type levels (Fig. 8C). These animals fail to develop trigeminal ganglia and are thought to lack cardiac trabeculae(28) which is the proposed cause of embryonic lethality. We have observed, however, that cardiac trabeculation can be seen in homozygous nulls (Fig. 9) and, in fact, the apparent lack of trabeculation is often seen in wild-type littermates indicating the lack of trabeculae may be simply an artifact of the dissection and fixation of the cardiac tissue.

We are also currently crossing the targeted animals into a homogeneous inbred genetic background (FVB/n), as it has been observed in the case of the EGF receptor knockout that genetic background profoundly affects the timing of its embryonic lethality(49, 50). We are hopeful of delaying the onset of embryonic lethality in order to better understand the underlying cause of the embryonic death. These knockout mice are currently 7 generations into the FVB background (the same strain we use for generating our transgenic animals), and soon we will test if timing of the lethal embryonic phenotype is the same as that observed previously by us and Lee et.al. in a mixed background.

NRG-immunoreactive proteins are subcellularly compartmentalized

We have recently observed through western blotting of IJ9921 cell extracts using a heregulin-specific antibody (HRG- α , Santa Cruz), that there are two "a"-immunoreactive forms of neuregulin synthesized in these cells, one of which is found only in the cytoplasm the other found only in the nucleus (Fig. 10). A cDNA expression library from this cell line has been made by packaging directionally cloned oligo-dT primed cDNAs in an expression vector (λ screen-Novagen). Using the antibody which recognizes the two proteins by Western blotting to screen 250,000 plaques, we isolated four clones which were immunoreactive through several rounds of plaque purification. Two of these clones have been sequenced, and neither appears to be a member of the neuregulin family. One clone is similar only to a human embryo expressed sequence tag (EST), and the other, as yet, has no significant similarity to anything in the sequence database.

Myc cooperates with NRG in tumor formation

Lastly, we have started to examine the ability of the *myc* gene product to cooperate with neu differentiation factor in accelerating tumor formation in bitransgenic animals expressing these genes from the MMTV promoter. Table I shows the phenotype(s) of fifteen bitransgenic animals generated to date in this study. Although the kinetics of tumor formation in these mice are not significantly different from those of the *myc* transgenic line alone, it is most noteworthy that ten of these fifteen mice are either virgin female or male animals: two populations not usually known to develop tumors in either line (though, occasionally, *myc* virgin females will develop a tumor between one and two years of age). It is interesting to note that, by Northern blot analysis, these tumors often express both transgenes but the NRG transgene is expressed at very low levels, if at all, and the histological phenotype is almost invariably Myc in nature. To determine if there is an inhibition of Myc-induced apoptosis as a result of NRG expression, we performed TUNEL assays on fixed paraffin-embedded sections from some of these tumors (Fig. 11). This assay detects DNA strand breaks, an indicator of programmed cell death. Myc-derived tumors are known to undergo some apoptosis (38), but we were somewhat surprised to see great amounts of apoptosis in the NRG tumors. Of possibly greater interest is that we have yet to observe signs of apoptosis in tumors derived from bitransgenic animals.

Conclusions

The *erbB2* gene has been shown to be transforming *in vitro* by overexpression, truncation, or point mutation. Our laboratory has demonstrated that the activating point mutation is transforming *in vivo* using a transgenic mouse model. The same mouse model has been used to show that the cellular form of *erbB2* (*c-neu*) is transforming as well, though with a slower onset of tumor formation. We initiated this study to determine if the same transgenic model could be used to assay the proliferative potential of Neu differentiation factor, a molecule known to stimulate the *erbB2* receptor through a heterodimeric receptor complex formed with other members of the EGF receptor family. *In vitro* experiments with NRG on different target mammary epithelial cell lines have yielded somewhat ambiguous results, though different responses to NRG may be a function of the complexity of the receptor types expressed on the surface of these cells. We hypothesized that if a normal receptor such as Neu, when overexpressed in the mammary gland, can provide an initiating event for tumorigenesis, then perhaps the overexpression of a gene product known to activate this receptor would have a similar function. The results of our experiments support this hypothesis in three ways. First, each animal in the study developed a mammary tumor; in some cases two or three independent tumors were found upon necropsy. Second, there is an incompletely penetrant phenotype in which there is a massive hyperplasia of the Harderian gland in approximately 50% of these mice. These hyperplastic and hypertrophic adenomas are benign in that they are non-invasive and they fail to grow when transplanted into syngeneic host animals. Clearly, elevated levels of NRG expression is not sufficient, as high levels of transgene expression are seen in the salivary gland, yet no histopathological consequences of this expression in that organ has ever been observed. The benign attributes of these growths imply that there are factors missing from the Harderian gland necessary for this hyperplasia to progress to a malignant neoplasm. Among these putative factors could be the right combination *erbB* receptor subtypes (*erbB1-4*). Interestingly, this Harderian gland phenotype has been seen in another transgenic mouse line in our laboratory, the MMTV-*ras* line, *ras* being a gene found to lie on the Neu signalling pathway. In that line, the glands are also hyperplastic and not neoplastic. Taken together with the NRG

transgenic animals, these data suggest that there may be factors downstream of Neu and *ras* which influence the susceptibility of a cell type to oncogenic transformation. A second, parallel, pathway may also provide the "second hit" necessary for oncogenesis and this pathway may be active in the mammary epithelium but inactive in the Harderian gland. It is also possible that there are factors inhibitory to the *neu-ras* signalling pathway which are present in the Harderian gland but absent in the mammary gland.

Lastly, a more subtle phenotype observed in the MMTV-NRG animals is that of the persistence of terminal end bud structures in the mammary gland of virgin females. The terminal end buds normally provide a source of differentiated myoepithelial cells at the growth points of the mammary ductal tree as it responds to mesenchymal signals to fill the mammary fat pad in the developing gland. These signals provide the TEBs with spatial and temporal growth cues, to avoid overgrowth of ductal structures as well as providing information to halt growth when the outer limits of the fat pad have been reached. Concomitant with the cessation of ductal growth, comes regression of the TEBs in a mature mouse. In our transgenic animals, however, the communication between the developing ductal structures and stromal signalling cells appears to be perturbed in that upon reaching the limits of the fat pad, TEBs do not consistently undergo the apoptotic regression seen in wild type animals. This is consistent with the observation of others that glial growth factor can inhibit apoptosis in Schwann cell cultures. Moreover, in mature transgenic females, TEBs are evident in regions of the mammary gland in which they are not normally seen, in the proximal regions of the gland (close to the lymph node in the #4 gland). There also appears to be a disruption in the signalling involving the direction of growth of the ducts, in that some ducts are found to have reversed direction and, in some cases, they have overlapped other ducts. These results suggest NRG expression overcomes inhibitory signals provided by the mammary stroma, not simply those signals which inhibit the growth of the ducts, but those which signal the direction of growth and those which influence the active regression of the terminal end buds. These potential inhibitory signals are overcome by a normal pregnancy, lactation and regression, as persistent TEBs are not observed in the mammary glands of such animals.

The second part of our work, discussed here, describes the embryonic lethality of generating a null mutation in the *c-neu* gene. It is obvious that this gene is

necessary for the proper development of the mouse, and we are currently searching for the mechanism by which the absence of Neu is lethal to the developing animal. Although others have recently published the targeted disruption of the *c-neu* gene, and suggest a lack of cardiac trabeculation leads to embryonic lethality, this is not consistent with our observations. It is possible however that these differences may be due to differences in gene targeting constructs, though we have seen no evidence of the production of a *c-neu* mRNA or a truncated Neu protein (data not shown).

Another part of our proposal concerns our desire to express NRG recombinantly to test its potential growth arresting effects on mammary carcinoma cell lines. We have been unable to generate sufficiently bioactive recombinant product, but we feel that we may be able to use the tumor cell line derived from our MMTV-NRG transgenic mouse line to purify such a product. We have used the established tumor cell line to examine an isoform of neuregulin not known to be associated with any specific biological function, the cytoplasmic domain of the "a" isoforms. We have isolated two cDNA clones from a library created from this cell line using an antibody which recognizes an epitope at the C-terminus of the NRG "a" isoforms. Unfortunately, sequence analysis of these clones has revealed neither to be a neuregulin isoform. The reasons for the antibody recognition of the gene products could be many, though it is likely the antibody, under the conditions we used, is not what the manufacturer purported to be—that is NRG-specific. It is also possible that these protein products share a common epitope with the neuregulin "a" form and it was merely coincidence that the antibody recognizes a different, compartmentalized, protein. It now remains to be decided if this phenomenon is worth further investigation.

Lastly, we have evidence to suggest that the neuregulin and c-myc transgenes can work together to alter the kinetics of tumor formation *in vivo*. Preliminary experiments point to the possibility that apoptotic induction by these transgenes in the tumors of transgenic animals may be inhibited when the transgenes are combined in a single tumor. These results are consistent with those observed by Amundadottir et al. with Myc and TGF α . Our laboratory is continuing to pursue this line of investigation.

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Figure Legends

Figure 1. MMTV-neuregulin Transgene Construct.

Above, a schematic representation of the MMTV-NRG transgene. The $\beta 2c$ isoform of neuregulin (NRG $\beta 2c$), amplified from a ras-transformed breast tumor, was fused to the mouse mammary tumor virus promoter (MMTV) followed by the simian virus 40 splicing and polyadenylation signals (polyA). Below is a Southern blot of mouse tail DNA restricted with BamHI and probed with a full length ^{32}P -labeled NRG cDNA. In addition to the endogenous NRG gene (high MW forms in both lanes), is a smaller ($\sim 2.1\text{kb}$) fragment in the second lane corresponding to the transgene, present as a single copy.

Figure 2. Northern Blot Analysis of Transgene Expression.

Ten μg total RNA from the tissues indicated were electrophoresed through a 1.2% agarose/formaldehyde gel. Hybridization was to a SV40 polyA-specific ^{32}P -labeled cDNA probe. Transgene-specific mRNAs are indicated by the arrow. The ethidium bromide stained gel is shown to demonstrate that the gel was evenly loaded.

Figure 3. Northern Blot Analysis of Harderian Gland Hyperplasias.

Ten μg total RNA from normal and hyperplastic Harderian gland from a male mouse (first two lanes) and mammary gland and hyperplastic Harderian gland from a female mouse (last two lanes) were analyzed by Northern blotting and probed as previously described.

Figure 4. Mammary Gland Whole Mounts.

Carmines red stained mammary gland whole mount preparations of 5 month old virgin wild-type (WT) and transgenic (TG) female mice, and regressed glands from one year old animals having undergone multiple rounds of pregnancy, lactation and regression (1 yr multiparous).

Figure 5. NRG-induced Tumor Onset.

Age of each animal, in days, at the appearance of a mammary gland tumor (x-axis), versus the number of tumor-free animals (y-axis). The median age at tumor onset was 357 days.

Figure 6. Northern Blot Analysis of Mammary Gland Tumors.

Ten μ g total RNA from normal mammary gland (MG), mammary gland tumors (TU) and affected Harderian gland (HG) were examined as described in previous figure legends.

Figure 7. Immunoblot Analysis of ErbB Receptor Proteins in a NRG-induced Mammary Gland Tumor Cell Line.

Total cell lysates or cell lysates immunoprecipitated with anti-Neu, ErbB3, or ErbB4 antibodies as indicated were run through a 5% SDS-PAGE gel. The gel was blotted to a nylon membrane and hybridized with the anti-phosphotyrosine antibody 4G10 (anti-P tyrosine, UBI), stripped and reprobed with the anti-Neu antibody (anti-Neu, Ab3, Oncogene Science) and stripped a second time and reprobed with an anti-ErbB4 antibody (anti-ErbB4, Santa Cruz). The NF tumor cell line was derived from a MMTV-*neu* oncogene-induced mammary gland tumor (Muller et al., 1988) and the NRG-induced tumor was established from the IJ9921 mammary gland tumor (see figure)>

Figure 8. Gene Targeting Construct, Southern Blot and Protein Levels.

(A) Schematic representation of the 1260 amino acid Neu protein (upper) and the 13 kbp genomic fragment (lower) used to generate the targeting construct. Triangles indicate introns. The shaded 2.8 kb region represents the genomic fragment replaced with a *neo^r* cassette. (B) Southern blot analysis of HindIII restricted genomic DNA probed with a cDNA probe found outside the targeting construct. (C) Western analysis of d10.5p.c. embryos homogenized in SDS-PAGE buffer and electrophoresed through a 5% gel and probed with a Neu-specific antibody.

Figure 9. Immunohistochemistry of Day 10.5 Neu Homozygous Null Mutant Embryo.

Fluorescent staining of the heart of a homozygous null (-/-) mutant *c-neu* knockout mouse with an anti-lectin antibody which labels endothelial cells. Arrow indicates trabeculae in the common ventricle (V). No such structures are seen in the common atrium (A).

Figure 10. Western Blot Analysis of Sub-cellular Fractions With "a" Isoform-specific Antibody.

Subcellular fractions of the IJ9921 cell line were analyzed by Western blotting. Above, an immunoblot of a 10% SDS-PAGE gel hybridized with the polyclonal antibody HRG- α (Santa Cruz). Below, the same fractions were analyzed with the anti-Neu antibody (Ab3) to demonstrate the nuclear fraction contained no membrane protein. The arrows show the two reactive bands found in either the nucleus or the cytoplasm.

Figure 11. TUNEL Assay.

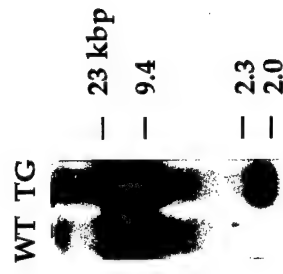
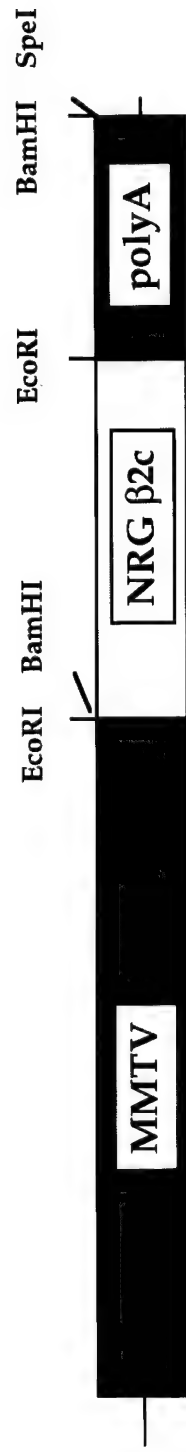
Using an *in situ* cell death detection kit from Boehringer Mannheim, DNA strand breaks were labeled using fluorescein-conjugated UTP and terminal transferase. A secondary antibody couple to alkaline phosphate with the appropriate substrate stains these strand breaks. Paraffin-embedded tissue sections used are as labelled. Sections were briefly counterstained with eosin.

Table I. Tumor onset in myc/hereregulin bitransgenic animals.

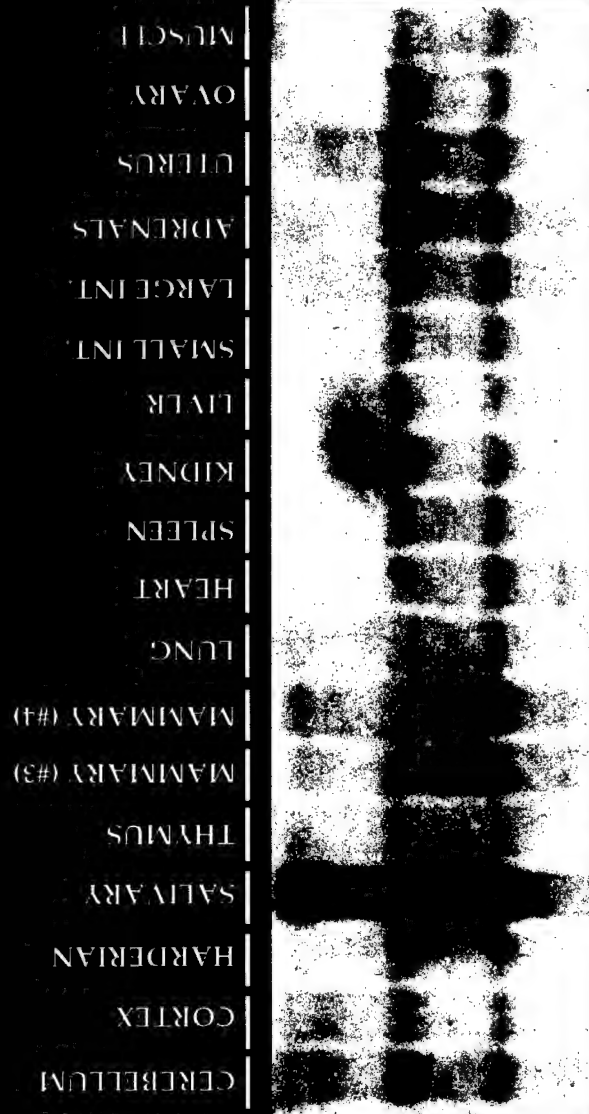
Myc x NDF#	age @ tu onset (mos)	sex (pregnancies)	tumor type
4239	8	M	n/d
4075	11	M	n/d
41896	12	M	myc
1011	10	M	myc
15967	10	M	myc
9031	3	F(1)	n/d
15946	4	F(++)	myc
4063	5	F(1)	myc
4064	5	F(1)	myc
4072	6	F(?)	myc
22	5	F(v)	myc
23	6	F(v)	myc
15963	5	F(v)	myc/NDF
15964	5	F(v)	myc
15968	6	F(v)	myc

Bitransgenic females were noted as virgin (v), multiparous (++), or having undergone a single (1) or unknown number of pregnancies (?). Tumor type was determined subjectively by our pathologists as being Myc or NDF-type tumors(24, 40).

NEUREGULIN TRANSGENE CONSTRUCT

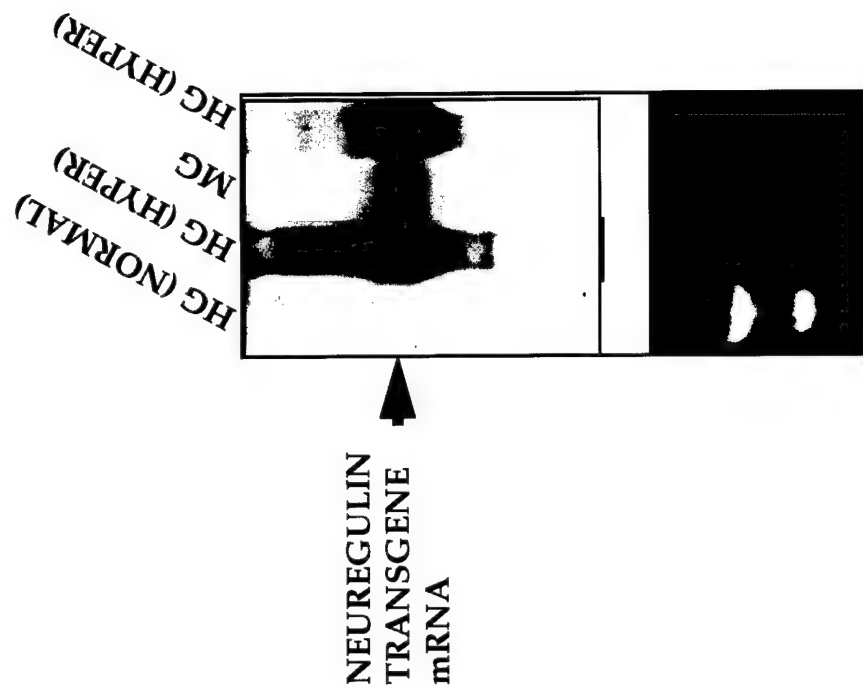


NEUREGULIN TRANSGENE TISSUE EXPRESSION



Mrane
Fig. 2

NEUREGULIN TRANSGENE OVEREXPRESSION IN AFFECTED HARDERIAN GLANDS



Trane
Fig 3

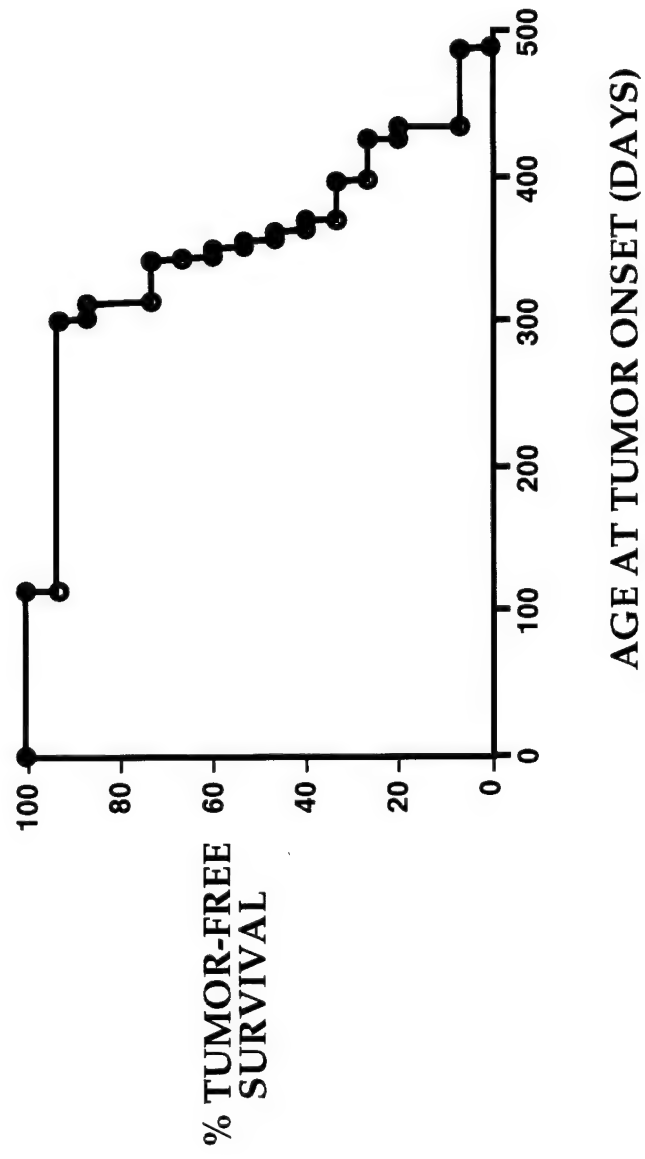
1 yr multiparous



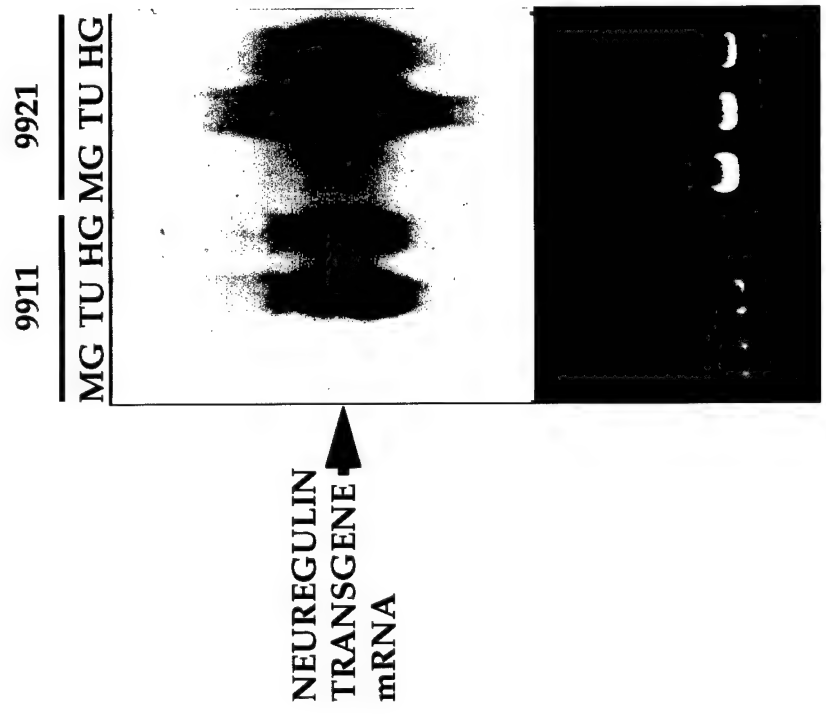
5 mo virgin



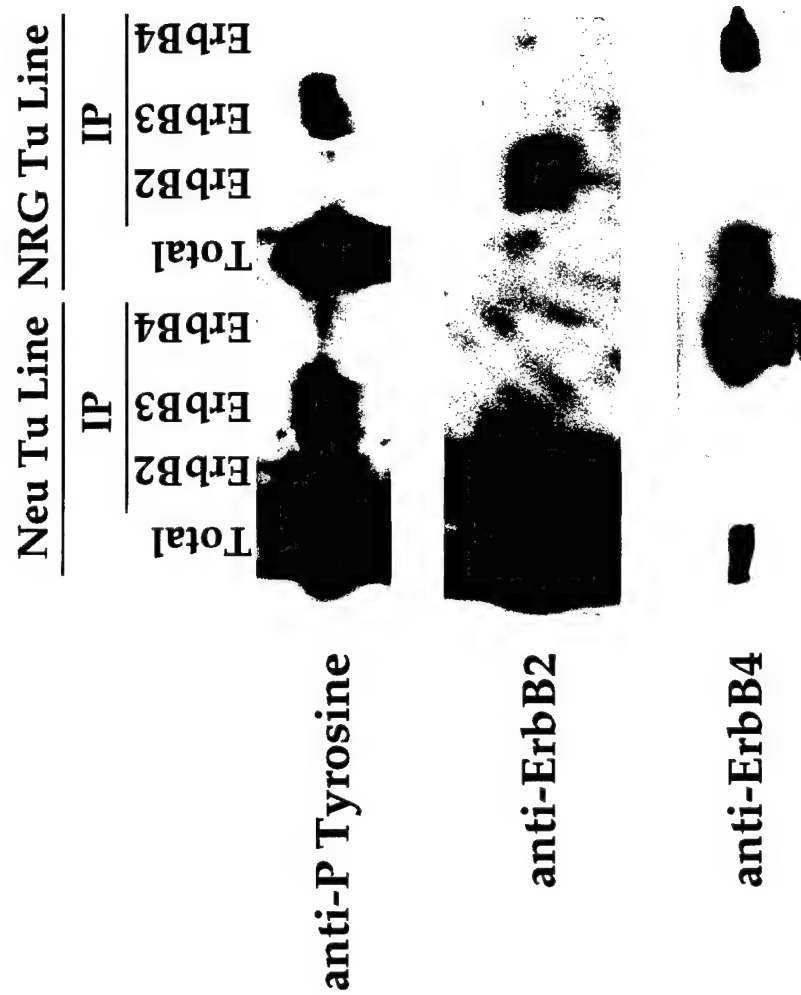
MMTV-NRG TUMOR ONSET



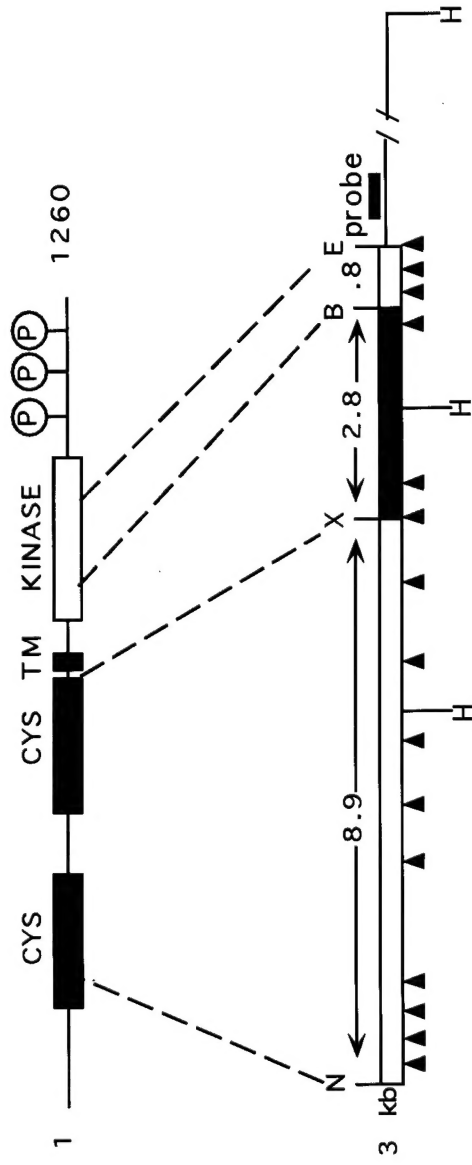
NEUREGULIN TRANSGENE EXPRESSION IN MAMMARY GLAND TUMORS



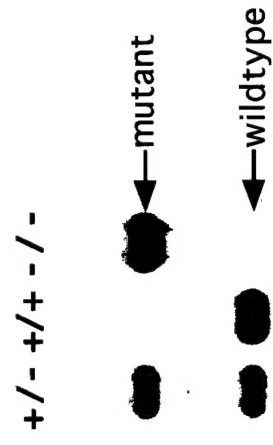
Krane
Fig. 6



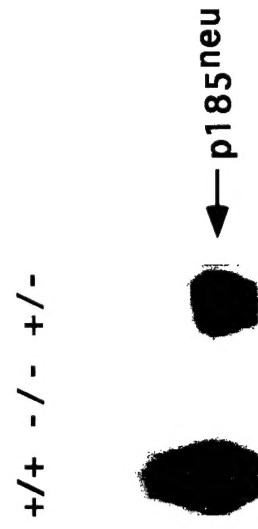
A.

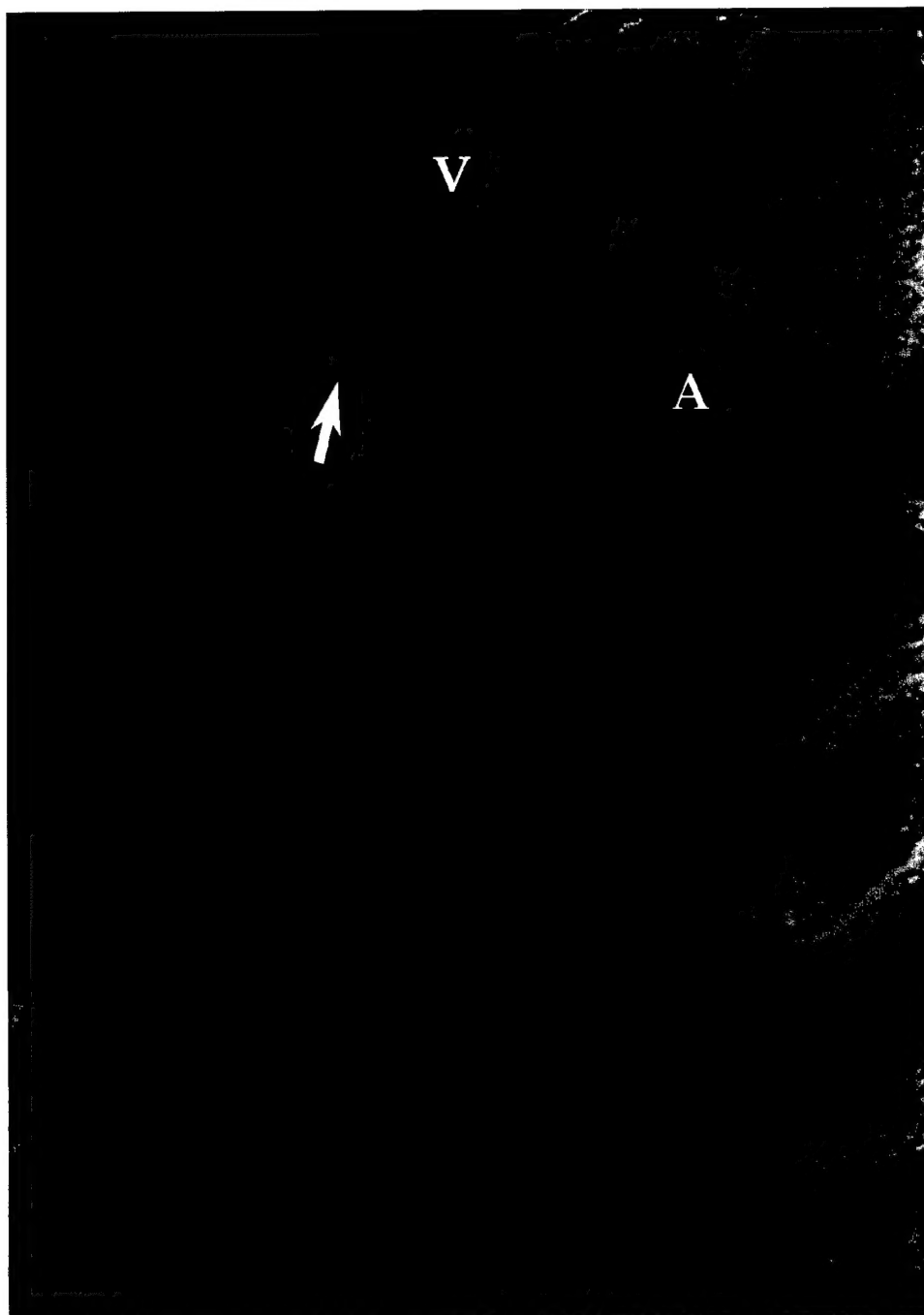


B.



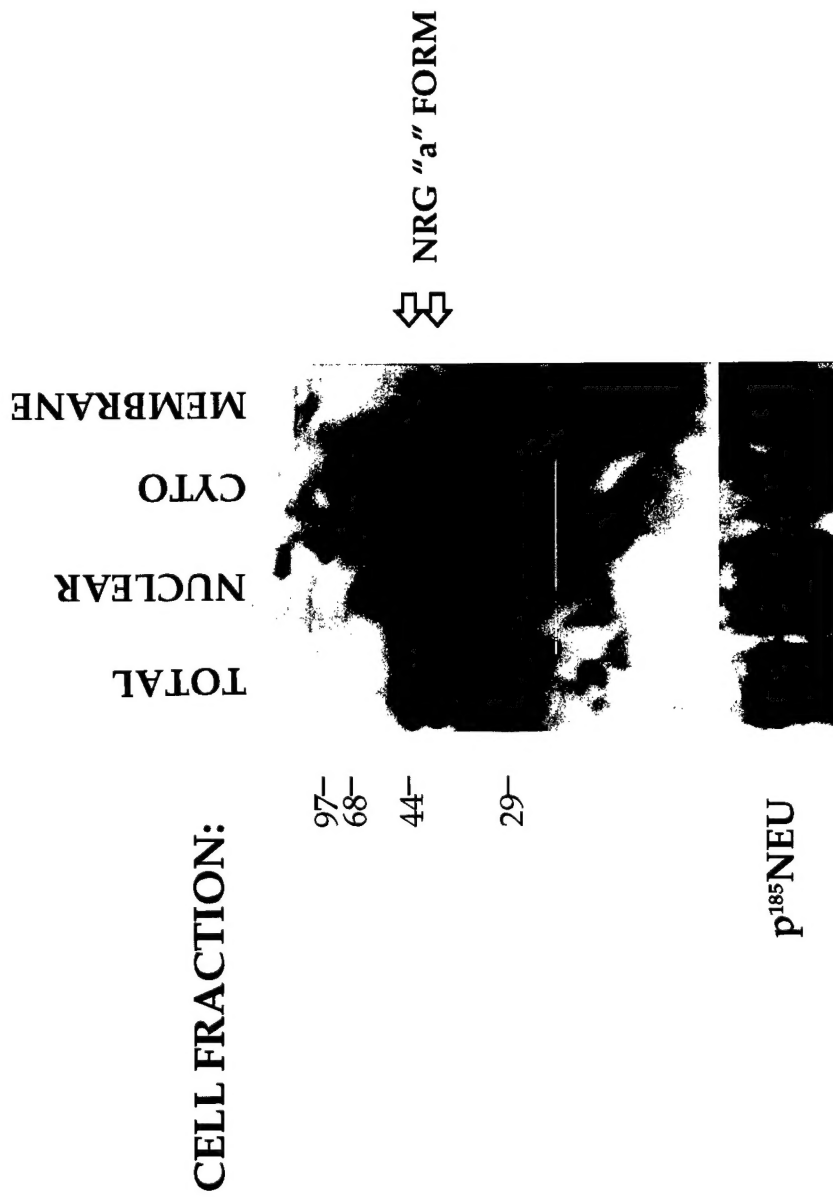
C.





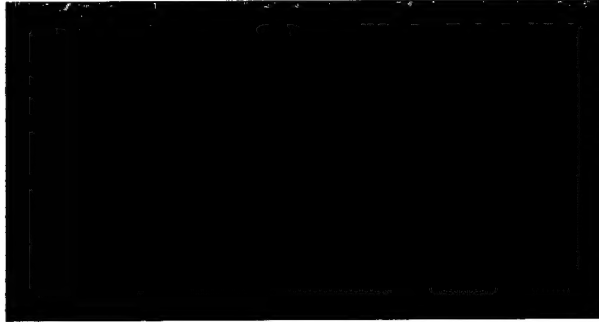
Krane
fig. 9

"a" ISOFORM EXPRESSION IN A NRG-INDUCED TUMOR CELL LINE

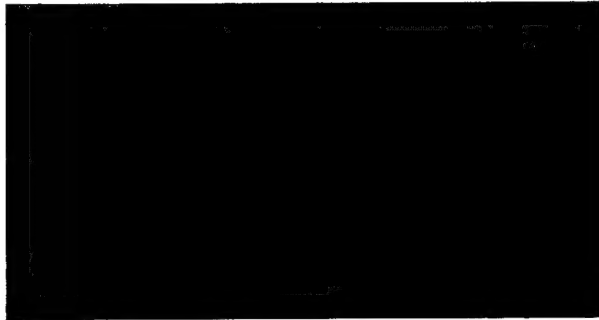


TUNEL ASSAY

Kidney



NRG Tu



Myc Tu



NRG/Myc Tu

